



# Spectrum of mutations in Italian patients with familial hypercholesterolemia: New results from the LIPIGEN study

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## Abstract

**Background:** Familial hypercholesterolemia (FH) is an autosomal dominant disease characterized by elevated plasma levels of LDL-cholesterol that confers an increased risk of premature atherosclerotic cardiovascular disease. Early identification and treatment of FH patients can improve prognosis and reduce the burden of cardiovascular mortality.

**Aim** of this study was to perform the mutational analysis of FH patients identified through a collaboration of 20 Lipid Clinics in Italy (LIPIGEN Study).

**Methods:** We recruited 1592 individuals with a clinical diagnosis of definite or probable FH according to the Dutch Lipid Clinic Network criteria. We performed a parallel sequencing of the major candidate genes for monogenic hypercholesterolemia (*LDLR*, *APOB*, *PCSK9*, *APOE*, *LDLRAP1*, *STAP1*).

**Results:** A total of 213 variants were detected in 1076 subjects. About 90% of them had a pathogenic or likely pathogenic variants. More than 94% of patients carried pathogenic variants in *LDLR* gene, 27 of which were novel. Pathogenic variants in *APOB* and *PCSK9* were exceedingly rare. We found 4 true homozygotes and 5 putative compound heterozygotes for pathogenic variants in *LDLR* gene, as well as 5 double heterozygotes for *LDLR/APOB* pathogenic variants. Two patients were homozygous for pathogenic variants in *LDLRAP1* gene resulting in autosomal recessive hypercholesterolemia. One patient was found to be heterozygous for the ApoE variant p.(Leu167del), known to confer an FH phenotype.

**Conclusions:** This study shows the molecular characteristics of the FH patients identified in Italy over the last two years. Full phenotypic characterization of these patients and cascade screening of family members is now in progress.

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**Keywords:** Familial hypercholesterolemia; *LDLR*; *PCSK9*; *APOB*; Pathogenic variants

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## 1. Introduction

At present three canonical genes underlie familial hypercholesterolemia (FH) with co-dominant transmission (autosomal dominant hypercholesterolemia, ADH): *LDLR* (ADH-type 1), *APOB* (ADH-type 2) and *PCSK9* (ADH-type 3). Other genes, like *STAP1* (ADH-type 4) and *APOE* (ADH-type 5) have been implicated in some rare forms of FH with dominant transmission [1–3]. In all molecular surveys performed so far in different cohorts of FH patients, mutations in *LDLR* gene were found in more than 85–90% of FH cases. More than 1700 mutations of this gene have been reported so far and annotated in the UCL database [4]. Few mutations in *APOB* underlie ADH-2, two of which are predominant [5]. Gain-of-function (GOF) mutations of *PCSK9* causing FH are exceedingly rare [6]. Only a single mutation in *APOE* gene [p.(Leu167del)] was found to be the cause of a dominant FH phenotype [3]. FH-like phenotypes with a recessive transmission are extremely rare. This group of disorders includes the classic autosomal recessive hypercholesterolemia (ARH) caused by mutations in *LDLRAP1* gene [7], sitosterolemia caused by mutations in *ABCG5/ABCG8* genes [8], and lysosomal acid lipase deficiency (LAL-D) due to mutations in *LIPA* gene [9].

With regard to the prevalence of FH with co-dominant transmission (ADH), recent data based on the determination of the carrier status of pathogenic variants in *LDLR* and *APOB* genes indicate a frequency of 1:217 for heterozygotes in the general population [10]. An extensive molecular study conducted in the Netherlands in genotyped FH patients indicated a prevalence of heterozygous ADH of 1:244 individuals and a prevalence of homozygous FH of 1:300,000 [11]. Several reports have shown that a variable percentage (20–40%) of patients with the clinical diagnosis of heterozygous FH do not carry mutations in the candidate genes mentioned above [12]. Some of these patients were found to carry a burden of LDL-cholesterol (LDL-C) increasing gene variants which cumulatively raise LDL-C levels into the heterozygous FH range [12,13]. However, there are patients in whom the polygenic effect does not explain the high LDL-C levels, indicating the possible presence of other genes yet to be identified [13].

In a previous study [14] we have characterized the molecular bases of ADH in a cohort of Italian FH patients attending three Lipid Clinics in Italy and performed a systematic analysis of the clinical features of mutation-positive ADH patients. This study paved the way for the construction of the LIPIGEN network, as an integrated network aimed at the early clinical/molecular identification and treatment of patients with genetic dyslipidemias (Ref. Averna M. et al. in this issue). The first aim of LIPIGEN was to improve the identification and molecular characterization of FH patients thanks to a collaboration among a large number of Lipid Clinics distributed over the entire country. In this brief report we show the results of the molecular characterization of more than 1000 patients

carried out during the first two years of activity of the LIPIGEN network.

## 2. Materials and methods

### 2.1. Subjects

For this analysis, 1592 unrelated patients from 20 outpatient clinics with clinical diagnosis of FH and a Dutch Lipid Clinic Network (DLCN) score  $\geq 6$  were genetically tested for the presence of variants in FH causing genes. Informed consent was obtained from all subjects. The project was approved by the Ethics Committees of each participating institution.

### 2.2. DNA isolation and sequencing

Genomic DNA was extracted using the Flexigene DNA kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. Appropriate primers were used for the PCR amplification of exons and exon-intron junctions of the following genes: *LDLR* (18 exons), *PCSK9* (12 exons) and *LDLRAP1* (9 exons), as well as regions in *APOB* exons 26 and 29 involved in the binding to LDLR, exon 4 of *APOE* gene and *STAP1* gene (9 exons). As internal control, regions of chromosome 21 were amplified. Fifty-eight regions of interest (20 Kb) were amplified from genomic DNA in five different multiplex PCR reactions. A second amplification was then performed in order to include an 8nt index sequence used for sample identification, as well as the adaptors used for sequencing on MiSeq (Illumina) equipment. Before sequencing, purified amplified samples were quantified and diluted to the same concentration. The genetic analysis, including the complete sequence of genes, was performed by Next Generation Sequencing (see above) followed by the Sanger sequence to confirm the presence of the identified variant. Multiplex Ligation-dependent Probe Amplification (MLPA) analysis was used to detect copy number variations.

*In silico* analysis of missense mutations was performed using PolyPhen-2 HumDiv and Hum Var (<http://genetics.bwh.harvard.edu/pph2/>), SIFT Human Protein (<http://sift.jcvi.org/>) and Mutation Taster ([www.mutationtaster.org/](http://www.mutationtaster.org/)). The potential effect of an intronic variant on pre-mRNA splicing was assessed by Human Splicing Finder (<http://www.umd.be/HSF3/HSF.html>), NetGene2 (<http://www.cbs.dtu.dk/services/NetGene2/>), BDGP Splice Site prediction ([http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html)), Splice Port (<http://spliceport.cbc.umd.edu/>) and Splice Site Score Calculation ([http://rulai.cshl.edu/new\\_alt\\_exon\\_db2/HTML/score.html](http://rulai.cshl.edu/new_alt_exon_db2/HTML/score.html)).

## 3. Results

In a series of 1592 FH index subjects, 1076 (67.6%) were found to carry at least one variant of the investigated

genes. Most of them (1016 subjects, 94.4%) carried at least one variant in the *LDLR* gene; a total number of 213 variants of *LDLR* gene have been identified. More than 90% of patients were carriers of definite pathogenic/likely pathogenic *LDLR* gene variants. Most *LDLR* variants were single nucleotide substitutions, mainly in the coding sequence. Large gene deletions were found in 53 subjects, whereas single nucleotide or minute deletions were found in 36 and 45 subjects respectively. Single nucleotide or minute duplications were found in 21 subjects.

The most common mutations in *LDLR* gene were [c.1646G>A, p.(Gly549Asp)] (107/1016), [c.662A>G, p.(Asp221Gly)] (84/1016), [c.1775G>A, p.(Gly592Glu)] (45/1016) and [c.1567G>A, p.(Val523Met)] (44/1016), all previously reported in Italian FH patients [14]. Thirty-two *LDLR* gene variants found in this survey had not been reported previously. *In silico* analysis indicated that 27 of

them could be regarded as pathogenic/likely pathogenic variants (Table 1).

Pathogenic *APOB* gene variants located in exon 26 (Table 2) were found in 34 subjects.

Four *PCSK9* variants were found in 36 subjects (Table 3). The most common were: i) the [c.137G>T, p.(Arg46Leu)] variant (found in 15 subjects), which is a known loss-of-function variant (LOF) associated with reduced LDL-C levels, and ii) c.60\_65dupGCTGCT [p.(Leu21tri)] (in 17 subjects), which is regarded to be a GOF variant and a possible cause of FH [6].

Twelve *LDLRAP1* gene variants were identified in 18 subjects. Five variants were pathogenic, 4 unlikely pathogenic and 3 of unknown significance (Table 4). Among the identified *LDLRAP1* variants, 4 have not been reported previously. However, only 1 of these new variants can be assumed to be pathogenic.

Table 1  
New *LDLR* gene variants.

| Gene variant (c.DNA)      | Type of variant             | Clinical significance                        | Protein change                 |
|---------------------------|-----------------------------|----------------------------------------------|--------------------------------|
| c.-97G>A                  | Promoter                    | Reduced transcription?                       |                                |
| c.3G>A                    | Aa change                   | Pathogenic                                   | p.(Met1Ile)                    |
| c.38_58del21              | Deletion                    | Pathogenic?                                  | p.(Ala13_Ala19del)             |
| c.68-?_190+?dup           | Exon 2 duplication          | Pathogenic                                   | p.(Gly24_Leu64dup)             |
| c.94T>C                   | Aa change                   | Pathogenic                                   | p.(Phe32Leu)                   |
| c.191-?_2311+?dup         | Exons 3–15 duplication      | Pathogenic                                   | p.[Ala771Val; Ser65_Ala771dup] |
| c.246delC                 | Deletion                    | Pathogenic                                   | p.(Cys82*)                     |
| c.313+4_313+16del13       | Affects donor splice site   | Pathogenic (new donor splice site in exon 3) | p.(Arg88Serfs*25)              |
| c.314-?_2583+?del         | Exons 4–18 deletion         | Pathogenic                                   | p.0                            |
| c.316_328delCCCAAGACGTGCT | Deletion                    | Pathogenic                                   | p.(Lys107Argfs*95)             |
| c.363C>A                  | Nonsense                    | Pathogenic                                   | p.(Cys121*)                    |
| c.620_626delGCGAGTG       | Deletion                    | Pathogenic                                   | p.(Gly207Alafs*56)             |
| c.641G>A                  | Aa change                   | Pathogenic                                   | p.(Trp214*)                    |
| C.688A>G                  | Aa change                   | Unlikely pathogenic                          | p.(Asn230Asp)                  |
| c.698T>C                  | Aa change                   | Unlikely pathogenic                          | p.(Val233Ala)                  |
| c.906C>A                  | Aa change                   | Pathogenic                                   | p.(Cys302*)                    |
| c.920A>C                  | Aa change                   | Pathogenic                                   | p.(Asp307Ala)                  |
| c.926C>A                  | Aa change                   | Pathogenic                                   | p.(Pro309His)                  |
| c.1037delT                | Deletion                    | Pathogenic                                   | p.(Leu346Argfs*24)             |
| c.1061-1G>T               | Acceptor splice site broken | Pathogenic (skipping Exon 8)                 |                                |
| c.1171delG                | Deletion                    | Pathogenic                                   | p.(Ala391Profs*22)             |
| c.1413_1414delAGinsGGACAT | Insertion/deletion          | Pathogenic                                   | p.(Gln474Hisfs*63)             |
| c.1470G>T                 | Aa change                   | Pathogenic                                   | p.(Trp490Cys)                  |
| c.1491delT                | Deletion                    | Pathogenic                                   | p.(Val498Serfs*9)              |
| c.1498delG                | Deletion                    | Pathogenic                                   | p.(Val500Leufs*7)              |
| c.1587-?_2583+?del        | Exons 11–18 deletion        | Pathogenic                                   | p.0                            |
| c.1686G>T                 | Aa change                   | Pathogenic                                   | p.(Trp562Cys)                  |
| c.1943_1944delCCinsG      | Deletion                    | Pathogenic                                   | p.(Ser48Cysfs*17)              |
| c.2120A>C                 | Aa change                   | Pathogenic                                   | p.(Asp707Ala)                  |
| c.2257C>G                 | Aa change                   | Unlikely pathogenic                          | p.(Pro753Ala)                  |
| c.2311_2311+15del16       | Donor splice site broken    | Pathogenic                                   |                                |
| c.2299A>G                 | Aa change                   | Possibly pathogenic                          | p.(Met767Val)                  |

*In silico* analysis of missense mutations was performed using PolyPhen-2 HumDiv and Hum Var (<http://genetics.bwh.harvard.edu/pph2/>), SIFT Human Protein (<http://sift.jcvi.org/>) and Mutation Taster ([www.mutationtaster.org/](http://www.mutationtaster.org/)). The potential effect of an intronic variant on pre-mRNA splicing was assessed by Human Splicing Finder (<http://www.umd.be/HSF3/HSF.html>), NetGene2 (<http://www.cbs.dtu.dk/services/NetGene2/>), BDGP Splice Site prediction ([http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html)), Splice Port (<http://spliceport.cbcb.umd.edu/>) and Splice Site Score Calculation ([http://rulai.cshl.edu/new\\_alt\\_exon\\_db2/HTML/score.html](http://rulai.cshl.edu/new_alt_exon_db2/HTML/score.html)).

Table 2  
Pathogenic variants in *APOB* gene.

| Gene variant (c.DNA) | Type of variant | Clinical significance | Protein change |
|----------------------|-----------------|-----------------------|----------------|
| c.10579C>T           | Aa change       | Pathogenic            | p.(Arg3527Trp) |
| c.10580G>A           | Aa change       | Pathogenic            | p.(Arg3527Gln) |
| c.10672C>T           | Aa change       | Pathogenic            | p.(Arg3558Cys) |

We found 17 subjects carrying two variants in *LDLR* gene (Table 5). Only 8 of these subjects carried five combinations of two variants with pathogenic effect. No formal family study was performed to assess whether the

two variants were on the same or on different alleles. However it can be reasonable assumed that carriers of two pathogenic variants (8 patients) might be compound heterozygotes.

Nineteen subjects were double heterozygotes for *LDLR*/*PCSK9* variants (Table 6). However only seven of these subjects were found to carry a putative or definite GOF variant of *PCSK9* expected to contribute to the FH phenotype. Five subjects were double heterozygotes for pathogenic variants of *LDLR* and *APOB* genes (Table 7).

We also identified 5 double heterozygotes for *LDLR*/*LDLRAP1* variants; all of them carried a pathogenic

Table 3  
Variants in *PCSK9* gene.

| Gene variant (c.DNA)             | Type of variant | Clinical significance                                      | Protein change |
|----------------------------------|-----------------|------------------------------------------------------------|----------------|
| c.137G>T                         | Aa change       | LOF variant <sup>a</sup>                                   | p.(Arg46Leu)   |
| c.-331C>A <sup>b</sup>           | Promoter        | GOF variant <sup>a</sup> ; 2,5-fold increase transcription |                |
| c.60_65dupGCTGCT (c.61_63triCTG) | Aa change       | GOF variant <sup>a</sup>                                   | p.(Leu21tri)   |
| c.1327G>A                        | Aa change       | Unlikely pathogenic                                        | p.(Ala443Thr)  |

<sup>a</sup> Dron JS, Hegele RA. Curr Opin Lipidol. 2017; 28: 161–9.

<sup>b</sup> Previously reported as -332C>A by Blesa S et al. J Clin Endocrinol Metab 2008; 93: 3577–83.

Table 4  
Variants in *LDLRAP1* gene.

| Gene variant (c.DNA)  | Type of variant             | Clinical significance | Protein change     |
|-----------------------|-----------------------------|-----------------------|--------------------|
| c.-17_-12dupGGCGGC    | —                           | Unknown significance  |                    |
| c.65G>A               | Nonsense                    | Pathogenic            | p.(Trp22*)         |
| c.89-1G>C             | Acceptor splice site broken | Pathogenic            | p.(Lys30Thrfs*3)   |
| c.457A>G <b>NEW</b>   | Aa change                   | Unknown significance  | p.(Met153Va)       |
| c.569G>C              | Aa change                   | Unlikely pathogenic   | p.(Gly190Ala)      |
| c.603dupC             | Duplication                 | Pathogenic            | p.(Ser202Leufs*19) |
| c.605C>A              | Aa change                   | Pathogenic            | p.(Ser202Tyr)      |
| c.653C>T              | Aa change                   | Unlikely pathogenic   | p.(Thr218Ile)      |
| c.748-2A>G <b>NEW</b> | Acceptor splice site broken | Pathogenic            | p.(Glu250Glyfs*4)  |
| c.811G>A              | Aa change                   | Unknown significance  | p.(Val271Ile)      |
| c.888C>G <b>NEW</b>   | Aa change                   | Unlikely pathogenic   | p.(Asp296Glu)      |
| c.896G>A <b>NEW</b>   | Aa change                   | Unlikely pathogenic   | p.(Gly299Asp)      |

Table 5  
Heterozygous carriers of two *LDLR* gene variants.

| <i>LDLR</i> gene 1st variant    | Protein                  | Clinical significance | <i>LDLR</i> gene 2nd variant | Protein                       | Clinical significance |
|---------------------------------|--------------------------|-----------------------|------------------------------|-------------------------------|-----------------------|
| c.1587-?_2583+?del (2 subjects) | p.0                      | Pathogenic            | c.1336C>G                    | p.(Leu446Val)                 | Unlikely pathogenic   |
| c.68-?_1845+?del                | p.(Val23Glyfs*29)        | Pathogenic            | c.2257C>G                    | p.(Pro753Ala) <b>NEW</b>      | Unlikely pathogenic   |
| c.1775G>A                       | p.(Gly592Glu)            | Pathogenic            | c.2299A>G                    | p.(Met767Val) <b>NEW</b>      | Unlikely pathogenic   |
| c.2072C>T                       | p.(Ser691Leu) <b>NEW</b> | Pathogenic            | c.38_58del21                 | p.(Ala13_Ala19del) <b>NEW</b> | Unknown significance  |
| c.352G>T                        | p.(Asp118Tyr)            | Pathogenic            | c.1211C>T                    | p.(Thr404Ile)                 | Pathogenic            |
| c.58G>A                         | p.(Gly20Arg)             | Unknown significance  | c.1474G>A                    | p.(Asp492Asn)                 | Pathogenic            |
| c.58G>A                         | p.(Gly20Arg)             | Unknown significance  | c.1257C>G                    | p.(Try419*)                   | Pathogenic            |
| c.662A>G                        | p.(Asp221Gly)            | Pathogenic            | c.698T>C                     | p.(Val233Ala) <b>NEW</b>      | Unlikely pathogenic   |
| c.665G>A                        | p.(Cys222Tyr)            | Pathogenic            | c.815A>C                     | p.(Asn272Thr)                 | Unknown significance  |
| c.858C>A                        | p.(Ser286Arg)            | Pathogenic            | c.2054C>T                    | p.(Pro685Leu)                 | Pathogenic            |
| c.898A>G (4 subjects)           | p.(Arg300Gly)            | Pathogenic            | c.907C>T                     | p.(Arg303Trp)                 | Pathogenic            |
| c.304C>T                        | p.(Gln102*)              | Pathogenic            | c.718G>A                     | p.(Glu240Lys)                 | Pathogenic            |
| c.418G>T                        | p.(Glu140*)              | Pathogenic            | c.352G>T                     | p.(Asp118Tyr)                 | Pathogenic            |

*In silico* analysis of missense mutations was performed using PolyPhen-2 HumDiv and Hum Var (<http://genetics.bwh.harvard.edu/pph2/>), SIFT Human Protein (<http://sift.jcvi.org/>) and Mutation Taster ([www.mutationtaster.org/](http://www.mutationtaster.org/)).

Table 6  
Double heterozygotes for *LDLR* and *PCSK9* gene variants.

| <i>LDLR</i> gene variant        | Protein                  | Clinical significance | <i>PCSK9</i> gene variant           | Protein                         | Clinical significance |
|---------------------------------|--------------------------|-----------------------|-------------------------------------|---------------------------------|-----------------------|
| c.1187-?_2140+?dup              | p.(Gly396_Thr713dup)     | Pathogenic            | c.1327G>A                           | p.(Ala443Thr)                   | Unlikely pathogenic   |
| c.1246C>T (2 subjects)          | p.(Arg416Trp)            | Pathogenic            | C.137G>T                            | p.(Arg46Leu)                    | LOF variant           |
| c.1257C>G                       | p.(Try419*)              | Pathogenic            | c.60_65dupGCTGCT<br>(c.61_63triCTG) | p.(Leu21tri)                    | GOF variant           |
| c.1257C>G                       | p.(Try419*)              | Pathogenic            | C.137G>T                            | p.(Arg46Leu)                    | LOF variant           |
| c.126C>A                        | p.(Try42*)               | Pathogenic            | C.137G>T                            | p.(Arg46Leu)                    | LOF variant           |
| c.1646G>A                       | p.(Gly549Asp)            | Pathogenic            | c.60_65dupGCTGCT<br>(c.61_63triCTG) | p.(Leu21tri)                    | GOF variant           |
| c.1783C>T                       | p.(Arg595Trp)            | Pathogenic            | c.60_65dupGCTGCT<br>(c.61_63triCTG) | p.(Leu21tri)                    | GOF variant           |
| c.1846-?_2583+?del (2 subjects) | p.0                      | Pathogenic            | C.137G>T                            | p.(Arg46Leu)                    | LOF variant           |
| c.2215C>T                       | p.(Gln739*)              | Pathogenic            | C.137G>T                            | p.(Arg46Leu)                    | LOF variant           |
| c.2312-3C>A                     | p.(Ala771_Ile796del)     | Pathogenic            | C.137G>T                            | p.(Arg46Leu)                    | LOF variant           |
| c.352G>T                        | p.(Asp118Tyr)            | Pathogenic            | c.60_65dupGCTGCT<br>(c.61_63triCTG) | p.(Leu21tri)                    | GOF variant           |
| c.373C>T (HO)                   | p.(Gln125*)              | Pathogenic            | C.137G>T                            | p.(Arg46Leu)                    | LOF variant           |
| c.418G>T                        | p.(Glu140*)              | Pathogenic            | c.60_65dupGCTGCT<br>(c.61_63triCTG) | p.(Leu21tri)                    | GOF variant           |
| c.418G>T                        | p.(Glu140*)              | Pathogenic            | C.137G>T                            | p.(Arg46Leu)                    | LOF variant           |
| c.662A>G                        | p.(Asp221Gly)            | Pathogenic            | c.-331C>A                           | 2,5-fold increase transcription | GOF variant           |
| c.788A>G                        | p.(Asp263Gly) <b>NEW</b> | Pathogenic            | c.60_65dupGCTGCT<br>(c.61_63triCTG) | p.(Leu21tri)                    | GOF variant           |
| c.1547G>A                       | p.(Gly516Asp)            | Unlikely pathogenic   |                                     |                                 |                       |

GOF: gain-of-function; LOF: loss-of-function.

*In silico* analysis of missense mutations was performed using PolyPhen-2 HumDiv and Hum Var (<http://genetics.bwh.harvard.edu/pph2/>), SIFT Human Protein (<http://sift.jcvi.org/>) and Mutation Taster ([www.mutationtaster.org](http://www.mutationtaster.org)).

Table 7  
Double heterozygotes for *LDLR* and *APOB* gene variants.

| <i>LDLR</i> gene variant | Protein                  | Clinical significance | <i>APOB</i> gene variant | Protein        | Clinical significance |
|--------------------------|--------------------------|-----------------------|--------------------------|----------------|-----------------------|
| c.1470G>T                | p.(Trp490Cys) <b>NEW</b> | Pathogenic            | c.10579C>T               | p.(Arg3527Trp) | Pathogenic            |
| c.1735G>A                | p.(Asp579Asn)            | Pathogenic            | c.10580G>A               | p.(Arg3527Gln) | Pathogenic            |
| c.662A>G                 | p.(Asp221Gly)            | Pathogenic            | c.10580G>A               | p.(Arg3527Gln) | Pathogenic            |
| c.662A>G                 | p.(Asp221Gly)            | Pathogenic            | c.10580G>A               | p.(Arg3527Gln) | Pathogenic            |
| c.662A>G                 | p.(Asp221Gly)            | Pathogenic            | c.10580G>A               | p.(Arg3527Gln) | Pathogenic            |

Table 8  
Double heterozygotes for *LDLR* and *LDLRAP1* gene variants.

| <i>LDLR</i> gene variant   | Protein                              | Clinical significance | <i>LDLRAP1</i> gene variant | Protein           | Clinical significance |
|----------------------------|--------------------------------------|-----------------------|-----------------------------|-------------------|-----------------------|
| c.858C>A                   | p.(Ser286Arg)                        | Pathogenic            | c.457A>G                    | p.(Met153Val)     | Unknown significance  |
| c.38_58del21+<br>c.2072C>T | p.(Ala13_Ala19del)+<br>p.(Ser691Leu) | Pathogenic            | c.653C>T                    | p.(Thr218Ile)     | Unlikely pathogenic   |
| c.682G>A                   | p.(Glu228Lys)                        | Pathogenic            | c.748-2A>G                  | p.(Glu250Glyfs*4) | Pathogenic            |
| c.1775G>A                  | p.(Gly592Glu)                        | Pathogenic            | c.811G>A                    | p.(Val271Ile)     | Unknown significance  |
| c.662A>G                   | p.(Asp221Gly)                        | Pathogenic            | c.888C>G                    | p.(Asp296Glu)     | Unlikely pathogenic   |

variant in *LDLR* gene, but only one was carrier of a pathogenic variant of *LDLRAP1* gene (Table 8).

Seven true homozygous patients were detected; 5 of them were homozygous for *LDLR* gene variants and 2 for *LDLRAP1* gene variants. (Table 9).

Finally, one subject was found to carry the *APOE* gene variant c.500\_502delTCC [p.(Leu167del)], known to be associated with a dominant FH phenotype.

#### 4. Discussion

In this preliminary report we describe the molecular findings in 1592 patients suspected to have definite or probable familial hypercholesterolemia identified through a national collaborative detection program. Only patients with a Dutch score  $\geq 6$  were admitted to the molecular investigation program. The exclusion of patients with



Table 9  
Homozygous subjects for *LDLR* and *LDLRAP1* gene variants.

| <i>LDLR</i> gene variant               | Protein                  | Clinical significance |
|----------------------------------------|--------------------------|-----------------------|
| c.1060+10G>A                           | ?                        | Unknown significance  |
| c.662A>G                               | p.(Asp221Gly)            | Pathogenic            |
| c.681C>G                               | p.(Asp227Glu)            | Pathogenic            |
| c.788A>G                               | p.(Asp263Gly)            | Pathogenic            |
| c.373C>T+(PCSK9 c.60_65dupGCTGCT, GOF) | p.(Gln125*)+p.(Leu21tri) | Pathogenic            |
| <i>LDLRAP1</i> gene variant            | Protein                  | Clinical significance |
| c.65G>A                                | p.(Trp22*)               | Pathogenic            |
| c.89-1G                                | p.(Lys30Thrfs*3)         | Pathogenic            |

possible FH (Dutch score 3–5) who might be carriers of pathogenic variants in FH candidate genes [11] was due to funding limitations, organization problems and to the deliberate intent to concentrate our efforts on patients who are more likely to have a monogenic disorder. We used targeted next generation sequencing which allowed the parallel sequencing of the major genes known to be responsible for monogenic forms of hypercholesterolemia.

The overall detection rate of pathogenic/likely pathogenic variants was around 60%, a figure which is comparable to that obtained in other studies [12,13]. The assignment of pathogenicity was based on *in silico* analysis using several algorithms [4] and information available in the published variant databases.

In accordance with findings from our previous study [14], more than 90% of variant-positive patients were carriers of pathogenic or likely pathogenic variants in the *LDLR* gene. Among these patients we discovered 4 true FH homozygotes carrying known pathogenic variants and 5 patients likely to be compound heterozygotes (as they were carriers of two known pathogenic or likely pathogenic variants of *LDLR* gene). In this survey we discovered 32 *LDLR* gene variants not reported previously in FH patients. A stringent *in silico* analysis [15] indicated that most of these variants can be assumed to be pathogenic or likely pathogenic.

As previously observed [14], only few patients were found to be heterozygous carriers of one of the three known *APOB* pathogenic variants. None of the patients was found to be homozygous/compound heterozygous for any of these variants. However some patients resulted to be double heterozygous for pathogenic *LDLR/APOB* variants. Overall, these findings confirm that *APOB* variants are a rare cause of FH in Italy.

Few patients were found to be heterozygous for putative pathogenic GOF variants of *PCSK9* gene. Surprisingly, none of them carried one of the three GOF variants reported previously in Italian FH patients [14] or one of those reported in a recent large survey of FH patients carrying *PCSK9* variants [16]. As a matter of fact, a convincing demonstration of pathogenicity of one putative GOF

variant found in our FH patients [c.60\_65dupGCTGCT (c.61\_63triCTG), p.(Leu21tri)] is not yet available. The same caution applies also to patients found to be double heterozygotes for *LDLR* and *PCSK9* variants (Table 6). Interestingly, most of these double heterozygous patients (carrying *LDLR* pathogenic variants) were found to carry the common polymorphic p.(Arg46Leu) variant, which is known to have an LDL-C lowering effect in normolipidemic as well as in FH patients [17–19].

We sequenced also the *LDLRAP1* gene whose pathogenic variants are the cause of autosomal recessive hypercholesterolemia (ARH), an exceedingly rare disorder in most countries with exception of the Italian island of Sardinia [7]. Two patients of our series were found to have ARH due to pathogenic variants of *LDLRAP1* while one patient was found to be double heterozygous for *LDLR* and *LDLRAP1* pathogenic variants.

This survey included also the sequence of *APOE* gene, in the light of previous studies suggesting that some rare apoE variants might be associated with an FH-like phenotype. We found only one patient heterozygous for a known apoE variant [p.(Leu167del)], considered the cause of FH with dominant transmission [3]. Recently a large study conducted in 228 Spanish FH patients in whom the *LDLR*, *APOB* and *PCSK9* pathogenic variants had been excluded, showed that nine patients (3.1%) were carriers of the p.(Leu167del) variant [20]. This variant was found to segregate with FH phenotype among index cases' family members. In view of these findings, *APOE* gene should be included among the genes responsible for FH [21].

This molecular survey paves the way to two main actions: i) a deep phenotypic analysis of the molecularly characterized patients, including response to treatment with hypolipidemic drugs; ii) implementation of the cascade screening of index patients' family members to identify carriers of pathogenic variants and characterize their phenotype. In view of our previous study, the cascade screening is expected to lead to the identification of at least 2000 carriers of pathogenic variants who may be unaware of the burden of having high LDL-C from birth and may benefit from early treatment and genetic counselling.

## Conflict of interest

The authors confirm that this article content has no conflict of interest.

## Appendix

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